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**EFFECTS OF VARIOUS HANDLING PROCEDURES
ON THE RESPONSES OF ZEBRA MUSSELS
(DREISSENA POLYMORPHA) IN BIOASSAY
TESTING**

AC Project No. 601C



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ON THE RESPONSES OF ZEBRA MUSSELS
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TESTING**

RAC Project No. 601C

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APRIL 1994



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EXECUTIVE SUMMARY

A series of experiments were conducted to determine the effects of maintenance method (fed or starved), stock location, season, mussel size, and rate of acclimation to temperature on the responses (mortality) of zebra mussels in bioassays. The data suggest that factors related to energetic demands of individuals (reproductive state, health, size) affect the responses of zebra mussels in bioassays. In addition, the patterns in the responses of zebra mussels were different depending on treatment (i.e., sodium hypochlorite or bayluscide). These differences are thought to be a function of both differences in behaviour (as with hypochlorite) or a function of the mode-of-action of the toxicant (as with bayluscide).

Mussels maintained on a diet of crushed *Chlorella* were more tolerant to bayluscide and more sensitive to sodium hypochlorite than were starved mussels. During the first ≈ 60 days in the laboratory, variability in LC50s for both hypochlorite and bayluscide were high suggesting that the mussels were physiologically adjusting to laboratory conditions. After the first ≈ 60 days, the resistance of mussels to both hypochlorite and bayluscide declined in relation to reductions in body condition. Both fed and starved mussels exhibited reductions in body health over the maintenance period (183 days) compared to the natural population in Lake St. Clair.

Zebra mussels collected during the early summer and late fall were more tolerant to both hypochlorite and bayluscide. Mussels collected during these times may be more resistant because of reduced metabolic demands during non-reproductive periods.

The tolerance of mussels collected from different locations varied with body size, as with bayluscide, and ash-free-dry weight (condition), as with hypochlorite. Smaller mussels may be more sensitive to bayluscide because of higher oxygen consumption rates compared to larger mussels. Mussels with a higher body condition may be more sensitive to hypochlorite because of higher energetic demands. Mussels with higher body condition may filter more water to obtain the food and energy required, increasing exposure to the biocide.

With mussels collected during the fall, there were no significant effects of mussel size on tolerance to hypochlorite or bayluscide. Effects of mussel size may be more important during the summer growing season when differences in oxygen consumption rates and other metabolic functions are at higher rates. During the fall, differences between small and large mussels may be undetectable because of more similar metabolic functions.

There were no significant differences in tolerances of mussels acclimated from 4 to 20°C at rates of 2 and 10°C per day. As such, it is possible to acclimate mussels from cold water to warm water within two days without influencing the results of bioassays.

These studies suggest that body size and condition, mussel stock, season, and laboratory protocols (fed or starved) have significant effects on responses of zebra mussels in bioassay testing. However, tolerances (LC50s) of zebra mussels to hypochlorite and bayluscide did

not vary by more than 2-3x under any situation. As such, range-finding tests can utilize zebra mussels that have been collected from any location and maintained in the laboratory under any protocol. However, to obtain more accurate estimates of the LC50, it is recommended that the tests be conducted in the first 12 days after collection, prior to physiological adaptation to laboratory stress, or between ≈ 30 and 60 days after collection, before reductions in body condition have substantial effects on the responses.

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The biofouling zebra mussel (*Dreissena polymorpha*) is thought to have entered the Great Lakes in 1985 (Hebert *et al.*, 1989) or 1986 (Griffiths *et al.*, 1991). It was first found in Lake St. Clair near Puce, Ontario, and has since spread into each of the Great Lakes with highest numbers and greatest prevalence in Lake Erie and Lake Ontario (Griffiths *et al.*, 1991). Since its introduction, it has impaired industrial (e.g., Lepage, 1993; Kovalak *et al.*, 1993) and recreational (Brousseau, 1992) uses of water resources, and has affected native fauna (particularly native bivalves; Mackie, 1991).

Currently, only sodium hypochlorite has temporary registration for use as a zebra mussel control agent (Claudi and Evans, 1993; Mondor, 1993) in Ontario. Chlorine is a very toxic, broad spectrum biocide (Becker and Thatcher, 1973), and is effective for controlling zebra mussels under a variety of implementation strategies (e.g., Klerks *et al.*, 1993; Van Benschoten *et al.*, 1993). Unfortunately, it combines with organic molecules to form persistent, bioaccumulative toxicants (C.C.M.E., 1987). Consequently, the use of chlorine for controlling zebra mussels in industrial facilities is of concern. The use of alternative, "safer", chemicals is preferred and a considerable amount of effort has been expended in the past to test new products.

Many groups in Canada and the U.S. (academic, government, and private business) are trying to find alternatives to chlorine for controlling zebra mussels, or are trying to refine the methodology with chlorine to minimize its use. At this point, there are no protocols for conducting toxicological work with zebra mussels. With the amount of work being conducted, it is vital that a standard protocol be set to make results comparable, and to allow effective application to large water distribution systems.

The objective of these studies was to examine potential sources of variation in the responses of zebra mussels in bioassays that have, as yet, been ignored. The potential sources of

variation examined in this series of tests included:

- season;
- geographic location;
- animal health (body condition);
- animal size (shell length); and,
- maintenance regime (fed vs starved; time maintained in a laboratory setting).

Mussel condition and metabolism is known to vary with season (Quigley *et al.*, 1993). If tolerance of zebra mussels to biocides is related to differences in body condition and/or metabolism, then differences in responses among seasons should be expected. Martin *et al.* (1993a,b) have suggested that zebra mussels in North America are more tolerant of sodium hypochlorite than mussels from Europe. Consequently, there is some suggestion that geographic stocks have different tolerances. If there are differences in tolerances among stocks in North America, then results from one group of investigators will not be comparable to results from another group if they have used separate stocks. Due to allometric relationships between gills or epithelium (i.e., potential routes of biocide uptake or impact) and body mass, smaller mussels should be more sensitive to biocides than larger mussels. However, some work with hypochlorite has suggested that this effect is minimal (Martin *et al.*, 1993a,b). This issue needs more attention because dosing regimes are designed, in part, on the assumption that smaller mussels are easier to kill (e.g., Claudi and Evans, 1993). Within the laboratory, maintenance protocols have the potential to affect physiology and health or condition (Stoeckmann and Garton, 1993). As indicated above, if the tolerances of mussels are related to health or physiology, then different laboratory maintenance protocols have the potential to affect responses of zebra mussels in bioassays.

Seasonal influences were examined by conducting bioassays with mussels collected throughout the ice-free season from Lake St. Clair at Puce, Ontario. The importance of stock location was determined by conducting bioassays with mussels collected from the St. Lawrence River (Cornwall), Hamilton Harbour, Lake Erie (Port Dover), Lake St. Clair

(Puce), and Lake Huron (Goderich). These sites maximized distance among the currently known stocks in Ontario. The effects of animal size were determined by comparing the tolerances of different size classes of mussels from one site (Lake St. Clair). The effects of maintenance procedures in the laboratory were determined by examining differences in the tolerances of mussels starved or fed a diet of crushed *Chlorella* over a period of six months.

Two reference toxicants were used throughout the studies to test the tolerance of zebra mussels to biocides. They were sodium hypochlorite and bayluscide (2',5-dichloro-4'nitrosalicylanilide). Sodium hypochlorite was selected because it is the only biocide registered for use in Ontario. Bayluscide was selected because it has a different mode-of-action than hypochlorite, and has been recently tested as a potential alternative biocide for controlling zebra mussels (Waller *et al.*, 1992; Fisher *et al.*, 1993). Chlorine toxicity can result from oxidation of gill membranes and blood proteins and interruption of osmoregulation. Oxidation of gill membranes results in gill damage with resulting decreased oxygen exchange capability and ultimately asphyxiation (Bass and Heath, 1977). With zebra mussels, Greenshields and Ridley (1957) suggested that the presence of chlorine impaired the ability of mussels to excrete ammonia, which is one mechanism used for regulation of osmotic pressures by this species.

The mollusacidal activity of bayluscide was first recognized by Gonnert and Schraufstatter (1958). It has been used to control intermediate snail hosts responsible for schistosomiasis (see references in Marking and Hogan, 1967) and as a synergist with TFM for controlling larval sea lampreys (NRCC, 1985). The mode-of-action of bayluscide is thought to be similar to that of TFM. At low concentrations, the presence of bayluscide in the water results in increased oxygen consumption. This increased oxygen consumption is thought to be a result of uncoupled oxidative phosphorylation (Kawatski *et al.* 1973; Ishak *et al.*, 1970), or the activation of an energy-requiring detoxification mechanism (Kawatski *et al.*, 1974). At high concentrations bayluscide is thought to inhibit glycolysis (Ishak *et al.*, 1970).

2.0 METHODOLOGY

2.1 Collections

All collections of zebra mussels for these studies were conducted in shallow water (1 m depth). Earlier work showed that attached mussels were 4-5 times more tolerant to bayluscide than unattached mussels (unpublished data). As such, it was necessary to use attached mussels for the remainder of the experiments. Mussels attached to small rocks (or other debris) were collected if available, because groups of 10-20 attached mussels were used during bioassays. In some cases, (e.g., Goderich), mussels attached to small rocks were not available. In such situations, mussels were scraped from the side of a dock into a 20-L pail and transported to laboratory. In the laboratory, these "unattached" mussels were given small rocks on which to attach. Within 3-4 days, any mussels that were unattached (most were already attached to other adults) became attached to small rocks and were ready to use for regular bioassays.

Mussels were transported to the laboratory in 20-L food-grade polyethylene pails. Water (≈ 60 L) from each collection site was also collected for culturing purposes. If the difference in air and water temperatures were $> 5^{\circ}\text{C}$, then mussels were transported in coolers to prevent rapid changes in water temperature during transport. For field collections that required transport times > 3 -4 hours, portable air bubblers were used to maintain oxygen levels, and zeolite rocks were used to maintain low ammonia levels.

2.2 Maintenance of Cultures

In the laboratory, daily records were kept for ammonia, pH, temperature, and dissolved oxygen. Using temperature, pH and ammonia concentrations, unionized-ammonia fractions were calculated based on OMOE (1984) equations. When concentrations of unionized ammonia approached 0.02 mg/L (recommended water quality guideline, OMOE, 1984)

culture water was changed.

All cultures were maintained at 20°C except when the experimental design required a different thermal regime. Each stock culture brought into the laboratory was maintained in 2-3 separate aquaria. This helped to ensure viable stocks, from specific collections, would be available if there were culturing difficulties in specific aquaria.

2.3 Tests Conducted

All bioassays were static, and were conducted at 20°C, using a dilution series that included five concentrations (500 ml solutions) plus a control for each of sodium hypochlorite and bayluscide. Bioassays were conducted using $\approx$ 10 mussels attached to small rocks. Due to loss of hypochlorite over time, concentrations were re-established every 24 h (see below).

During the tests, mortality was assessed every 24 hours (for nine days) for tests with sodium hypochlorite, and was assessed at 3, 6, 12, 24, 48, 72, and 96 hours for tests with bayluscide. Mortality was assessed by gently prodding the siphonal region of the mussels if the shells were gaping. Mussels were given a score depending on their response: (1) shell closed (= alive); (2) shell open but closes when the siphonal region is prodded (= alive); (3) shell open, cannot close upon prodding, but small muscular contractions in the epithelium of the siphonal region, or circulation of water into or out of the incurrent or excurrent siphons (= alive); and, (4) shell gaping with none of the above signs of life (= dead). These assessments were done using a binocular microscope at 6-12x magnification. Death of mussels was confirmed by putting "dead" mussels into "recovery" chambers with fresh dilution water.

Mussels exposed to relatively higher concentrations of bayluscide (42-56 $\mu\text{g/L}$) often ($\approx$ 10% of the time) closed their shells without withdrawing their siphons. The reason for

this response was not determined, however, in cases when the extruded siphon responded to prodding the mussel was considered alive. When the extruded siphon did not respond, the mussel was considered dead.

After each test, mussels from the untreated (control) chambers were measured (shell length) and preserved in 70% ethanol. Representative mussels were processed for determination of weight of soft tissues. Weight of soft tissues relative to shell length can be used as a measure of body condition. To determine weight of soft tissues, mussels were dried at 50°C for 48 hours, weighed, ashed at 550°C for two hours and reweighed. The difference between the two weights approximates the weight of the soft tissues (= Ash-Free-Dry Weight; AFDW), although the weight of the periostracum (protein layer on the outer surface of the shell) usually accounts for a low percentage in bivalves (e.g., McCall, 1988).

2.3.1 Dilution Series With Bayluscide

It was difficult to establish a dilution series for bayluscide that would provide partial mortalities. Initially, a logarithmic series was used which included 3.5, 7, 14, 28, and 56 $\mu\text{g/L}$. The only concentration that provided a partial mortality was 28 $\mu\text{g/L}$. Concentrations in later tests were adjusted to improve the resolution of the tests (i.e., provide a better estimate of the LC50). The dilution series that consistently provided a good (more reliable) estimate of the LC50 included concentrations of 21, 28, 35, 42, and 56 $\mu\text{g/L}$. With this dilution series, partial mortalities were usually observed at 28, 35, and 42 $\mu\text{g/L}$, 100% mortalities were usually observed at 56 $\mu\text{g/L}$, and 100% survival was usually observed in the controls and at 21 $\mu\text{g/L}$. Confirmation of chemical concentrations of bayluscide are expensive ($\approx$ \$400.00 per determination) and were not conducted. Some of the variation in the LC50s observed in these studies, therefore, may be due to variation in the stock concentrations.

New stock solutions of bayluscide were prepared to 100 mg/L on the same day that tests were started because there is some indication that bayluscide degrades rapidly (NRCC, 1985).

2.3.2 Dilution Series With Sodium Hypochlorite

The dilution series with sodium hypochlorite which consistently provided partial mortalities included target concentrations of 1, 2, 4, 6, and 8 mg/L (as total residual oxidant [TRO]). Over a 24 hour period, these concentrations typically declined, but the decline was not linear: most of the loss of TRO occurred during the first few hours. The area under this curve could be used to estimate the average exposure over the 24 hour period, as long as the concentration after 24 hours was consistent. However, this was not the case. For example, after 24 hours, TRO concentrations in the 8 mg/L treatment containers varied anywhere from 0 to 4 mg/L. To estimate the daily average TRO exposure for each container, a preliminary experiment to describe the TRO loss curve for each target concentration was conducted. The curve showed that after 24 hours, the daily average concentration (i.e., the average of the initial + final concentration) was overestimating the "true" daily average exposure. Multiplying the average of the initial and final concentrations by 0.4 at 1 and 2 mg/L, by 0.65 at 4 mg/L, and by 0.73 at 6 and 8 mg/L was a better approximation of the daily average exposure to hypochlorite. As such, these conversion factors were used to determine the daily average exposure concentrations which were used as the "actual" test concentrations in the bioassays. It was considered important to do this because it made the LC50 values more conservative (i.e., they were lower [half] have been if the target concentration had been used).

2.3.3 Dilution Water

Dilution water was dechlorinated City of Toronto tap water. That water was used because

it has chemistry representative of water from the Great Lakes (Table 1).

2.3.4 Effects of Maintenance Method

To determine if zebra mussels can be maintained in a laboratory for extended periods of time without having adverse effects on responses in bioassays, mussels were collected from Lake St. Clair (Puce) on June 3, 1992. Half of the mussels collected on that date were not fed (starved). The remaining mussels were fed crushed *Chlorella* every second day (fed). Bioassays with hypochlorite and bayluscide were conducted after 5, 12, 26, 33, 59, 88 and 183 days, using both fed and starved mussels. Dried, crushed *Chlorella* was used because it has been suggested that as the easiest means of providing digestible food for zebra mussels (Nichols, 1993).

2.3.5 Effects of Season

To determine if mussels collected at different times of the year would respond differently (more tolerant or sensitive) in bioassays, mussels were collected from Lake St. Clair on five separate occasions (June 3, 9, July 18, August 6, 26). Bioassays with hypochlorite and bayluscide were conducted within one week of collection.

2.3.6 Effects of Stock Location

To determine if mussels collected from different locations would respond differently in bioassays, mussels were collected from populations located in the St. Lawrence River (across from Cornwall just upstream of the Reynolds Metals Co.), Hamilton Harbour, Lake Erie (at Port Dover), Lake St. Clair (at Puce), and Lake Huron (at Goderich). The collections were made during the week of August 24, 1992. All mussels were collected from

the nearshore zones in < 1 m of water. Bioassays with hypochlorite and bayluscide were conducted on August 31, 1992.

2.3.7 Effects of Mussel Size

Mussels collected from Puce on October 8 had two size classes of mussels (4-6 and 10-15 mm). To determine if there were significant differences in responses of these two size classes, bioassays with bayluscide and sodium hypochlorite were conducted. Concentrations were prepared in duplicate with ≈ 10 mussels of each size class in each replicate. Generally, mussels from the smaller size class were attached to mussels from the larger size class. As such, both size classes had to be tested in the same containers to ensure that mussels from the smaller size class were attached.

2.3.8 Effects of Acclimation Rate

To determine if acclimation rate had an effect on the responses of mussels in a bioassay, mussels were collected from Lake St. Clair on November 10 when water temperatures were 4-5°C. Mussels were transported in a cooler because air temperatures were $\approx 10^\circ\text{C}$ on that day. In the laboratory, mussels were divided into two groups which were put into aquaria in separate water baths (both baths were preset at 4°C). One water bath was used to raise temperatures at a rate of 2°C per day, whereas the other was used to raise temperatures at a rate of 10°C per day. Effectively, raising temperatures at a rate of 10°C per day is equivalent to letting the water warm up naturally to ambient laboratory temperatures: after 24 hours, temperature of the 10°C water bath was 15°C, and after 48 hours it was 20°C. Bioassays with bayluscide and sodium hypochlorite were conducted after the mussels in the slower acclimation rate had been acclimated to 20°C.

2.4 Statistical Analyses

Estimates of the LC50s were made using either Probit Analysis (when possible) or Spearman-Kärber calculations (Hubert, 1984). To determine if two LC50s were significantly different, Sprague and Fogels (1977) calculations were used.

To determine if variations in condition or shell length of zebra mussels were related to LC50 values, linear regressions were used with the LC50 as the dependent variable (Y) and shell length or condition as the independent variable (X). Comparisons were made among all available data points, and within specific groups of mussels (e.g., mussels collected on the same date, from different locations, or mussels collected on different locations but on the same date).

As indicated above, tissue weight (Ash-Free-Dry Weight) was used as a measure of body condition. However, to properly infer the "condition" of a mussel, it is necessary to adjust the tissue weights by size (shell length) so that they are relative among animals. To do this, analysis of covariance (ANCOVA) was used with tissue weight as the dependent variable (Y), shell length as the covariate, and mussel group (a combination of time period and stock) as the independent variable (X) (Snedecor and Cochran, 1989). The MGLH module of SYSTAT provides Least-Squares-Means for each level of the classification variable (in this case mussel group), which are the tissue weights adjusted for differences in shell length. The average shell length of all mussels used in this analysis was 1.21 cm. As such, the tissue weights reported in other sections of the report refer to the expected tissue weight if all the mussels were 1.21 cm long.

3.0 RESULTS

3.1 Effects of Maintenance Method

Variations in the LC50s of fed and starved mussels treated with sodium hypochlorite and bayluscide are shown in Figures 1, and 2. Significant variation in the LC50s for both hypochlorite and bayluscide over the maintenance period (183 days) are shown. However, differences in the LC50s between fed and starved mussels were infrequent. Variations in the LC50s of mussels treated with hypochlorite were significantly negatively correlated with shell length such that smaller mussels (≈ 1.0 cm) tended to be more tolerant of hypochlorite than larger mussels (1.2-1.3 cm). There was no relationship between the LC50 of mussels treated with hypochlorite and body condition, and the LC50s of mussels treated with bayluscide were not correlated with either body condition or shell length.

Average shell length and AFDW of mussels used in bioassays varied with maintenance time (Figures 3, 4). There were no consistent differences in shell length of fed and starved mussels over the maintenance period, however, mussels in Lake St. Clair tended to be larger than mussels maintained in the laboratory (Figure 3). There were no significant differences in the AFDW of fed and starved mussels maintained in the laboratory, however, AFDW of both groups tended to decrease with time in the laboratory. Mussels from Lake St. Clair tended to have higher AFDW than either fed or starved mussels (Figure 4).

3.2 Effects of Season

The LC50s of zebra mussels treated with hypochlorite and bayluscide followed a similar pattern: LC50s were lowest in early summer (June 9), increased during the summer, and were highest during the fall (Figures 5, 6). The LC50 of mussels treated with hypochlorite was not significantly correlated with either body condition or shell length, however, the LC50s of mussels treated with bayluscide were significantly positively correlated with

variations in shell length ($r = 0.565$, $p = 0.052$; Figure 7).

Mussels collected from Lake St. Clair tended to increase in shell length over the study period (Figure 3), whereas, AFDW tended to remain constant (Figure 4).

3.3 Effects of Stock Location

There were significant variations in the LC50s of mussels collected from different locations and treated with hypochlorite and bayluscide (Figures 8, 9). With hypochlorite, LC50s were lowest for mussels collected from Hamilton Harbour (1.23 mg/L) and highest for mussels collected from the St. Lawrence River at Cornwall (1.95 mg/L). Variations in the LC50s of mussels treated with hypochlorite were significantly negatively correlated with body condition ($r = -0.778$, $p = 0.031$, Figure 8) such that mussels with a higher condition from Lake St. Clair and Hamilton Harbour were more sensitive to hypochlorite than mussels with a lower condition. With bayluscide, mussels collected from Lake Huron at Goderich were the most sensitive (LC50 = 28 $\mu\text{g/L}$), whereas, mussels collected from Hamilton Harbour were the most tolerant (LC50 = 49 $\mu\text{g/L}$). Variations in the LC50 of mussels treated with bayluscide were significantly positively correlated with shell length ($r = 0.555$, $p = 0.092$, Figure 9) such that smaller mussels (1.2-1.3 cm) were more sensitive to bayluscide than larger mussels (1.8 cm).

3.4 Effects of Mussel Size

There were no significant differences in the LC50s of large and small mussels collected in October and exposed to either sodium hypochlorite or bayluscide. For large (10-15 mm) mussels exposed to hypochlorite, the LC50 was 1.61 mg/L (95% confidence limits: 1.14, 2.29), whereas for small (4-6 mm) mussels the LC50 was 1.13 mg/L (0.75, 1.60). For large mussels treated with bayluscide the LC50 was 38.9 $\mu\text{g/L}$ (35.8, 42.3), whereas the LC50 for

smaller mussels was 44.1 (39.2, 47.8).

3.5 Effects of Acclimation Rate

There were no differences in the LC50s of mussels acclimated at 2°C or 10°C d⁻¹ when exposed to either sodium hypochlorite or bayluscide. For mussels acclimated at 10°C d⁻¹ the LC50 for bayluscide was 42 µg/L (42-56), whereas for mussels acclimated at 2°C d⁻¹ the LC50 was 46 µg/L (42-50) (Table 2). For mussels acclimated at 10°C d⁻¹, the LC50 for sodium hypochlorite was 2.11 mg/L (1.28-2.24), whereas for mussels acclimated at 2°C d⁻¹ the LC50 was 1.64 mg/L (1.16-2.09).

4.0

DISCUSSION

The data suggest that factors related to energetic demands of individuals (reproductive state, health, size) affect the responses of zebra mussels in bioassays. In addition, the patterns in the responses of zebra mussels were different depending on treatment (i.e., sodium hypochlorite or bayluscide). These differences are thought to be a function of both differences in behaviour (as with hypochlorite) or a function of the mode-of-action of the toxicant (as with bayluscide).

4.1

Effects of Maintenance Method

The health of both fed and starved zebra mussels declined with time in the laboratory (Figure 4) suggesting that the maintenance regimes were not adequate for maintaining mussel health. Mussels health may have declined as result of an inappropriate feeding schedule or because temperatures were too high, or both. Reductions in AFDW were greatest for mussels maintained on the diet of crushed *Chlorella* suggesting that they were expending more energy trying to obtain and digest the food than the starved mussels were expending just maintaining body tissues. Stoeckmann and Garton (1993) have shown that when mussels are fed in excess of some "optimal" amount, body condition and ability to develop gametes becomes impaired. The results of their studies suggest that the feeding regime utilized in this study may have been providing *Chlorella* in amounts exceeding the "optimal" level resulting in poor body condition. In addition, Stoeckmann and Garton (1993) suggest that the optimal temperature for growth and condition of zebra mussels is near 15°C. Consequently, it may be easier to maintain health of mussels with reduced water temperatures.

There was significant variation in responses in bioassays during the first ≈60 days of maintenance in the laboratory. For both fed and starved mussels treated with bayluscide or hypochlorite, responses tended to decline during the first ≈30 days, increase during the

next ≈ 30 days, and decline throughout the remainder of the maintenance period. After the first ≈ 60 days, reductions in tolerance appeared to be related to changes in AFDW. These variations in tolerances of zebra mussels to toxicants may be related to physiological and behavioral changes that are occurring during the first ≈ 50 days of captivity. Data from Sprung (1991), Sprung and Borcharding (1991), and Bielefeld (1991) suggest that during starvation events, zebra mussels change their physiology and selectively utilize energy from various tissue stores.

In general, the variations in the LC50s over time are similar to what is predicted by the General Adaptation Syndrome (Selye, 1946). In response to stress (in this case laboratory conditions) animals go through an initial alarm phase during which physiological mechanisms are elevated, followed by a resistance stage during which physiological mechanisms return to normal function, and finally an exhaustion stage. The initial alarm stage in this case may have resulted in the initial decline in tolerance of zebra mussels to the biocides. The resistance stage may have occurred after this with resistance returning to nearly normal (initial) values at ≈ 30 -60 days. The exhaustion stage may have been point at which the tolerance of zebra mussels is significantly lower than the initial values (after ≈ 60 days).

Mussels maintained on the diet of crushed *Chlorella* were consistently more tolerant to bayluscide and more sensitive to hypochlorite than starved mussels, particularly after the initial ≈ 60 -day period (Figures 1, 2). The increased sensitivity of fed mussels to hypochlorite suggests that either an increased tendency to filter water (increasing exposure to hypochlorite) or a reduction in condition was responsible. Presumably, mussels which have been fed would be more likely to filter water than mussels which have not been fed. In general, zebra mussels can detect and close their shells in the presence of high concentrations of sodium hypochlorite (Van Benschoten *et al.*, 1993). An increased tendency of fed mussels to filter during exposure to hypochlorite might explain their increased sensitivity.

The increased tolerance of fed mussels to bayluscide may be a function of an increased ability of the fed mussels to detoxify the compound. Although the mode-of-action of bayluscide is not exactly known, its general effect is to exhaust the animal by interrupting oxygen transfer and energy producing mechanisms (NRCC, 1985). Detoxification mechanisms of fed mussels may be "turned on" as a result of a higher rate of energy use, or as a result of a requirement to detoxify compounds associated with the food.

The diet of crushed *Chlorella* did not provide the nutrition required by the mussels. However, it is doubtful that a replacement diet or a change in overall maintenance (e.g., the water composition recommended by Sprung, 1987) would be able to provide the nutrients that would result in more consistent results in a bioassay, particularly during the first ≈ 60 days. The variations in LC50s observed during the first ≈ 60 days occurred independently of changes in mussel condition, and appear to reflect adaptation of the mussels to laboratory conditions (see discussion of General Adaptation Syndrome above). Some of the factors in the laboratory which differ compared to the real environment and which may result in short-term (i.e., during the first ≈ 60 days) physiological changes in the mussels include: (1) photoperiod; (2) daily thermal fluctuations; and, (3) differences in water quality (e.g., relatively higher concentrations of ammonia or other waste products). It may be useful to examine these effects to determine if it is possible to control some of the variation observed during early periods in the maintenance program.

Mussels collected June 9 were significantly more sensitive to hypochlorite than mussels collected June 3 (Figure 6). The difference between the two results was probably the protocol used to conduct the bioassay. In all of the other tests, mussels were collected and tested after at least four days in the laboratory. However, the mussels collected on June 9 were tested the day after being collected. This suggests that mussels tested immediately after bringing them to the laboratory are more sensitive than they are if they are given 4-5 days to adjust to laboratory conditions. The increased sensitivity during the first few days may be related to the shock of sampling, transport and laboratory acclimation or exposure to elevated levels of ammonia during the first few days of laboratory maintenance. After

most collections were brought to the laboratory, water in the maintenance aquaria, generally, required replacement due to high ammonia (> 2.6 mg/L) levels. If adaptation to initial shock does occur, then it would suggest that there are two adaptation periods for the mussels: (1) during the first 1-4 days after arrival into the laboratory; and, (2) during the first ≈ 60 days.

The variations in LC50s for mussels treated with both hypochlorite and bayluscide did not vary by more than 2-3x throughout the complete maintenance period. As such, mussels maintained for extended periods of time may be suitable for conducting toxicity tests depending on the resolution required. However, for more accurate prediction of a consistent LC50, it may be necessary to conduct bioassays within the first ≈ 12 days of collection or between ≈ 30 -60 days after collection.

4.2 Effects of Season

Mussels collected during the early summer and late fall were more tolerant of both bayluscide and hypochlorite than mussels collected during the middle of the summer (Figures 5, 6). These variations in tolerance may be a function of variations in mussel size, health, behaviour, or reproductive state. The LC50 of mussels treated with bayluscide was significantly related to mussel shell length, however, this effect is confounded with season because of the growth of mussels at Lake St. Clair during the summer (Figure 3). Mussels collected during the fall and early summer may be more tolerant to biocides because they have a lower metabolic rate during those periods (Quigley *et al.*, 1993) which should result in a reduced filtration rate and less exposure to the toxicants. Lower energetic demands during these periods may be related to reproductive events. Mackie (1993) showed that mussels at the Puce site in Lake St. Clair actively reproduce between late June to early July and late October to early November, which coincides with periods of increased metabolic rates described by Quigley *et al.* (1993).

Studies that are examining the efficacy of new products for control of the zebra mussel should use stocks collected during the early summer (prior to June) or in the late fall (after September) which tend to be more tolerant to biocides. These stocks will provide more liberal (higher) estimates of the amount of biocide required to control an infestation.

4.3 Effects of Stock Location

There was significant variation in the tolerances of mussels from different stocks when treated with bayluscide and hypochlorite. However, there were also significant differences in shell length and AFDW of mussels from the various locations. For mussels treated with hypochlorite, the LC50 was related to body condition (AFDW) (Figure 8). For mussels treated with bayluscide, the LC50 was related to shell length (Figure 9).

Mussels with a higher condition may have been more sensitive to hypochlorite because of a higher filtration rate. Presumably, mussels with a higher body condition would require more energy to maintain tissues and would filter at a higher rate to obtain the food and energy needed. When treated with hypochlorite, this may result in more exposure than for mussels that have a lower condition.

For mussels treated with bayluscide, mussels with a greater size may be more resistant than smaller mussels because of differences in metabolic demands. Quigley *et al.* (1993) have indicated that large mussels have a lower rate of oxygen consumption than smaller mussels. With bayluscide, this should be very significant, because the overall effect of bayluscide is to impair oxygen utilization (NRCC, 1985).

With bayluscide, the Hamilton Harbour stock appears to have influenced the relationship between tolerance and shell length, and removing it suggests that the effect of size on tolerance to bayluscide was negligible (Figure 9). Factors other than size may have been responsible for the increased tolerance of the Hamilton Harbour stock including a greater

ability to detoxify compounds or tolerance to stressful laboratory conditions due to historical exposure to contaminants or poor water quality. The Hamilton Harbour stock could be assumed to have been historically exposed to the most degraded water quality. During collections, ammonia levels at Hamilton Harbour were higher than at any of the other sites (0.9 mg/L at Hamilton Harbour, 0.4-0.5 mg/L at all other sites). Poulton *et al.* (1986) showed that ammonia levels coming out of the harbour are often in excess of 1.5 mg/L. These factors may make the Hamilton Harbour stock more tolerant of contaminants (i.e., improved detoxification mechanisms) or more tolerant of poor water quality (i.e., higher ammonia levels) in the laboratory, both resulting in higher a better ability to detoxify bayluscide than the other mussel stocks.

These results indicate that studies that are examining the efficacy of new products for control of zebra mussels should report the condition of the stock sampled so that results from other studies can be more accurately compared. If the objective of bioassay testing is to determine the levels of a chemical for control of a specific stock, then testing should be conducted with the stock of interest. If the objective is to compare results of bioassays among studies, then use of a common stock may be useful. The use of a common stock has been recommended by some U.S. workers trying to standardize test results (R. McMahon, The University of Texas at Arlington, pers. comm.).

4.4 Effects of Mussel Size

The results indicate that the influence of mussel size on tolerance to biocides depends on season, or the effect may be negligible. With mussels collected during the fall (October 8) from Lake St. Clair there were no differences in tolerances of large (10-15 mm) and small (4-6 mm) mussels to either hypochlorite or bayluscide. During late summer (August 24), smaller mussels from Lake Huron and Lake St. Clair were shown to be more sensitive to bayluscide than larger mussels from Hamilton Harbour suggesting a significant size effect. The difference between the two sets of data may have been a function of the time of year

mussels were collected and their associated physiological (reproductive) condition. Data in Quigley *et al.* (1993) suggest that all size classes of mussels collected during the fall should have similar metabolic rates, presumably because reproductive events have slowed down. Similarities in tolerances of large and small mussels during the fall may be related to this phenomenon. Differences in tolerances of large and small mussels during the summer may be related to differences in metabolism or reproduction. However, as indicated above, the effect of size on the tolerance of various stocks to bayluscide (Figure 9) was somewhat dubious because the larger mussels from the Hamilton Harbour stock may be more conditioned for detoxifying contaminants and be more tolerant.

Other studies have shown that size effects are significant but there is evidence that size effects are manifested only when considering different life stages. Martin *et al.* (1993a,b) showed that size effects, although significant, explain little of the variation (16-26%) in results of hypochlorite toxicity tests with zebra mussels. In most other studies that suggest there is a size effect, reference is usually made to differences between larval and adult zebra mussels. According to Fisher *et al.* (1993) and Kilgour (1993), post-veligers tend to be more sensitive to toxicants than both adults and veligers when non-oxidizing chemicals are tested. Van Benschoten *et al.* (1993) and Fisher *et al.* (1993) suggest that when oxidants are used, veligers are the most sensitive life stage. As such, the lack of large size effects in this study may be a result of having examined the tolerances of adult mussels only and not other life stages (veligers, post-veligers).

4.5 Effects of Acclimation Rate

There were no significant differences in the LC50s of mussels acclimated to 20°C at 2 or 10°C per day. McCauley (Wilfred Laurier University, pers. comm.) has found similar results during studies of thermal tolerance of zebra mussels. As such, it is possible to raise temperatures of stock cultures rapidly without significantly affecting the results of a bioassay. This is important, because it may be more useful to maintain zebra mussel stocks

at 10 or 15°C (to optimize condition), but conduct tests at some higher temperature (e.g., 20°C; to simulate summer temperatures). In addition, if collecting mussels during the fall or winter, these results suggest that it is possible to acclimate the mussels within only a few days. As such, more experiments can be conducted within a given time frame.

5.0

CONCLUSIONS AND RECOMMENDATIONS

The data suggest that factors related to energetic demands of individuals (reproductive state, health, size) affect the responses of zebra mussels in bioassays. In addition, the patterns in the responses of zebra mussels were different depending on treatment (i.e., sodium hypochlorite or bayluscide). These differences are thought to be a function of both differences in behaviour (as with hypochlorite) or a function of the mode-of-action of the toxicant (as with bayluscide).

Mussels maintained in the laboratory, at 20°C, on a diet of crushed *Chlorella* exhibited reductions in health and tolerance to biocides. Studies should be conducted to determine if maintaining mussels at lower temperatures and under different feeding schedules are more appropriate and can maintain health and tolerance of mussels over longer periods.

Mussels maintained on a diet of crushed *Chlorella* were more sensitive to hypochlorite, possibly because they were conditioned to feeding (filtering water) resulting in an increased exposure to the biocide. Mussels maintained on a diet of crushed *Chlorella* were more tolerant to bayluscide, possibly because detoxification mechanisms were "turned on". Feeding status of mussels should be considered if using laboratory stocks for biocide testing.

Mussels were more sensitive to biocides immediately after collection from the natural environment compared to five days after they were been brought to the laboratory. Tolerance of mussels to biocides increased during the first ≈ 30 days and decreased from ≈ 30 to 60 days. These variations were thought to be due to physiological adjustments that were being made by the mussels to laboratory conditions. After ≈ 60 days in the laboratory, tolerances of mussels decreased in relation to reductions in body condition. The LC50s of mussels maintained in the laboratory tended not to vary more than 2-3x compared to initial (day 4-5) values. As such, mussels can be maintained in the laboratory for extended periods of time and still provide estimates of the LC50 which are within 2-3x of the true value. However, to obtain more accurate estimates of the "true" LC50, mussels should be tested

before within the first ≈ 12 days or between ≈ 30 and 60 days.

Mussels collected during the early summer and late fall were more tolerant of both bayluscide and hypochlorite than mussels collected during the middle of the summer. Studies that are examining the efficacy of new products for control of the zebra mussel should use stocks collected during the early summer or late fall because they will give more liberal (higher) estimates of the amount of biocide needed for control.

The responses of mussels collected from different locations varied with body size (as with hypochlorite) and body condition (as with bayluscide). Differences among stocks should, therefore, be comparable if data on mussel size and condition are reported. For control of specific stocks, testing should be conducted with the specific stock in question. To compare efficacies of various biocides, the use of a common stock may be warranted. However, if the use of common stocks is implemented, then handling protocols should also be reported.

Effects of mussel size on responses in bioassays may be significant if the stock is sampled during the summer when physiological mechanisms are operating at higher rates than during the fall. Effects of size may be more pronounced when comparing different life stages (i.e., adults *vs* veliger larvae or postveligers) than when comparing different sizes of adults.

Zebra tolerances of mussels acclimated quickly ($10^{\circ}\text{C d}^{-1}$) and slowly (2°C d^{-1}) from 4 to 20°C were not significantly different. Consequently, mussels can be acclimated from cool temperatures to warmer temperatures at rates at least as fast as $10^{\circ}\text{C d}^{-1}$ without affecting results in a bioassay. Given this result, it should be possible to maintain mussels at lower temperatures to prevent reductions in body condition, and conduct tests at higher temperatures to simulate summer conditions.

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Table 1. Chemistry of dilution water used for the bioassays. Units are mg/L unless otherwise indicated. Data courtesy of OMOE (Mr. D. Poirier, pers. comm.).

Parameter	Value
copper	0.0012
nickel	<0.001
lead	<0.005
zinc	<0.002
iron	0.082
cadmium	<0.002
chromium	<0.001
chloride	30.80
sulphate	33.32
total kjeldahl nitrogen as N	0.30
total ammonium as N	0.10
total nitrates as N	0.45
nitrite as N	0.005
dissolved organic carbon	1.3
dissolved inorganic carbon	20.4
conductivity, $\mu\text{mho}/\text{cm}$ at 25°C	345
total hardness	137.1
calcium	40.4
magnesium	8.80
sodium	15.4
potassium	1.78
total alkalinity	91.5
pH	8.21
fluoride	1.12

Table 2. Variations in the sodium hypochlorite and bayluscide LC50s for mussels acclimated to 20°C from 4°C at rates of 2 and 10°C day⁻¹. 95% confidence limits are given in parentheses.

Biocide	Acclimation Rate	
	2°C day ⁻¹	10°C day ⁻¹
Hypochlorite (mg/L)	1.64 (1.16, 2.09)	2.11 (1.28, 2.24)
Bayluscide (µg/L)	46 (42, 50)	42 (42, 56)

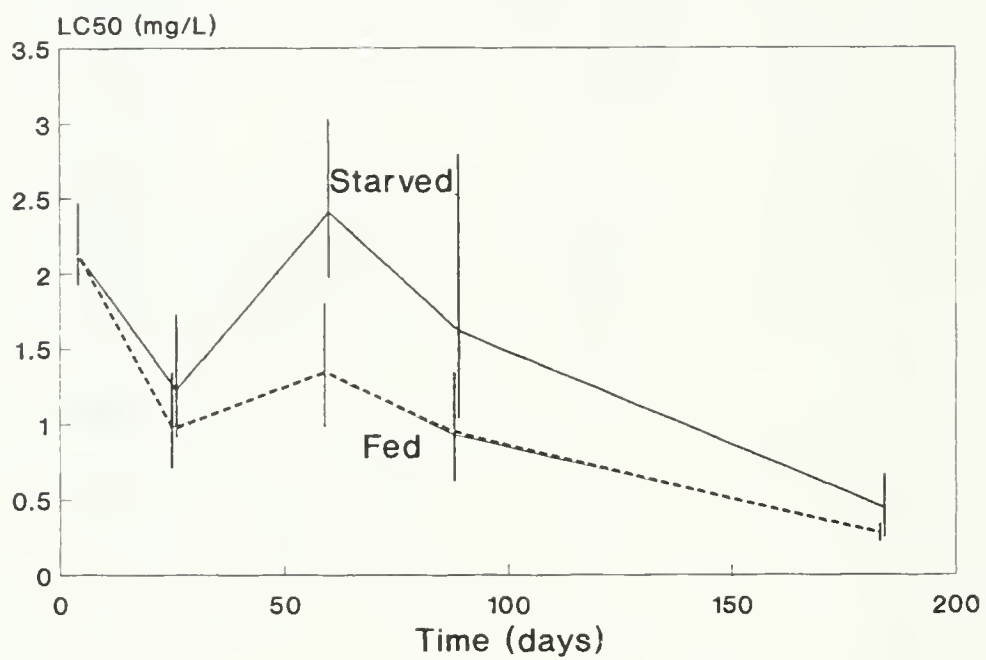


Figure 1. Variations in the sodium hypochlorite LC50 of fed and starved mussels maintained in the laboratory for up to 183 days.

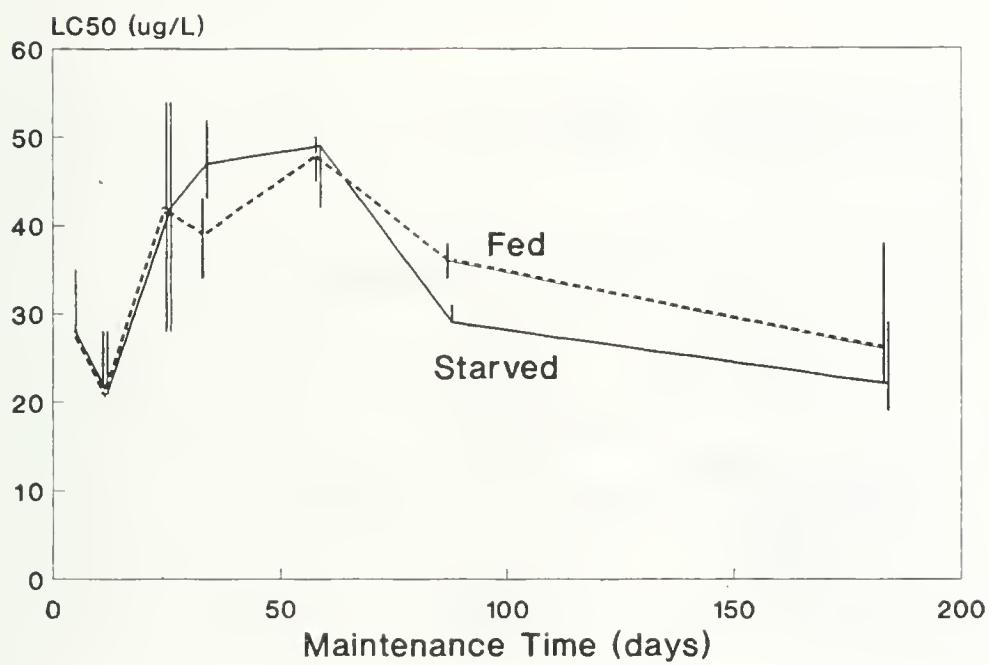


Figure 2.

Variations in the bayluscide LC50 of fed and starved zebra mussels maintained in the laboratory for up to 183 days.

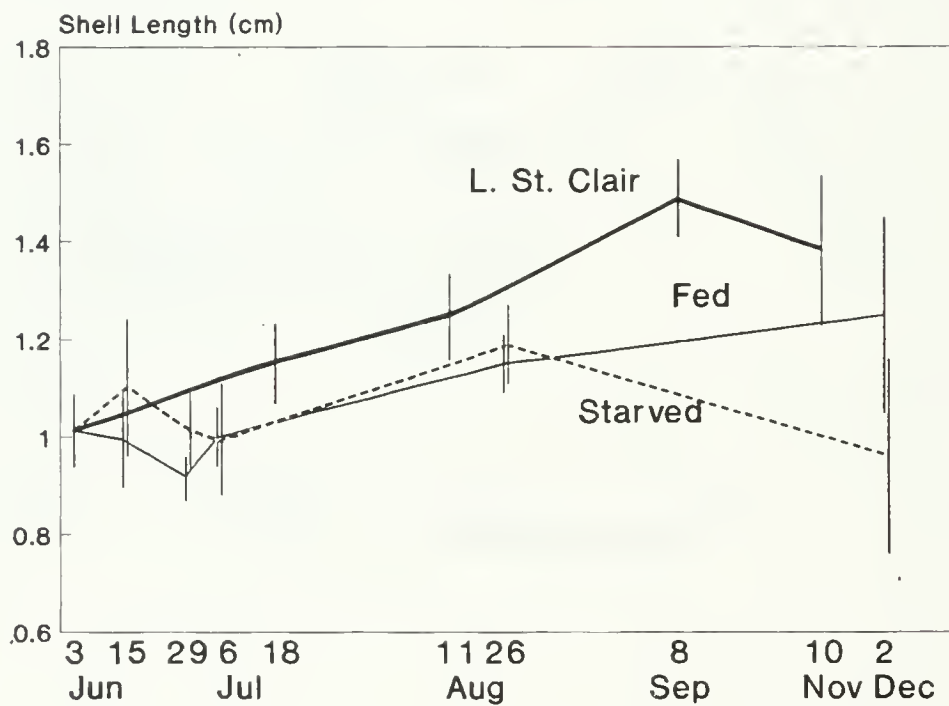


Figure 3.

Variations in shell length of fed and starved mussels maintained in the laboratory and mussels collected from Lake St. Clair on a seasonal basis.

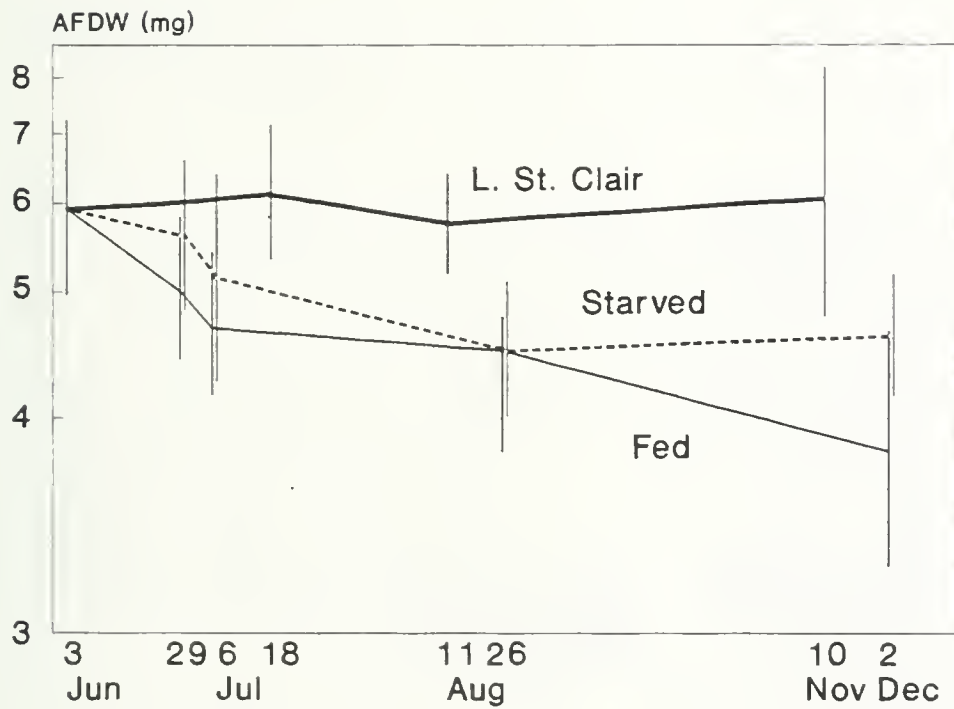


Figure 4.

Variations in Ash-Free-Dry-Weight (AFDW) of fed and starved mussels maintained in the laboratory and mussels collected from Lake St. Clair on a seasonal basis.

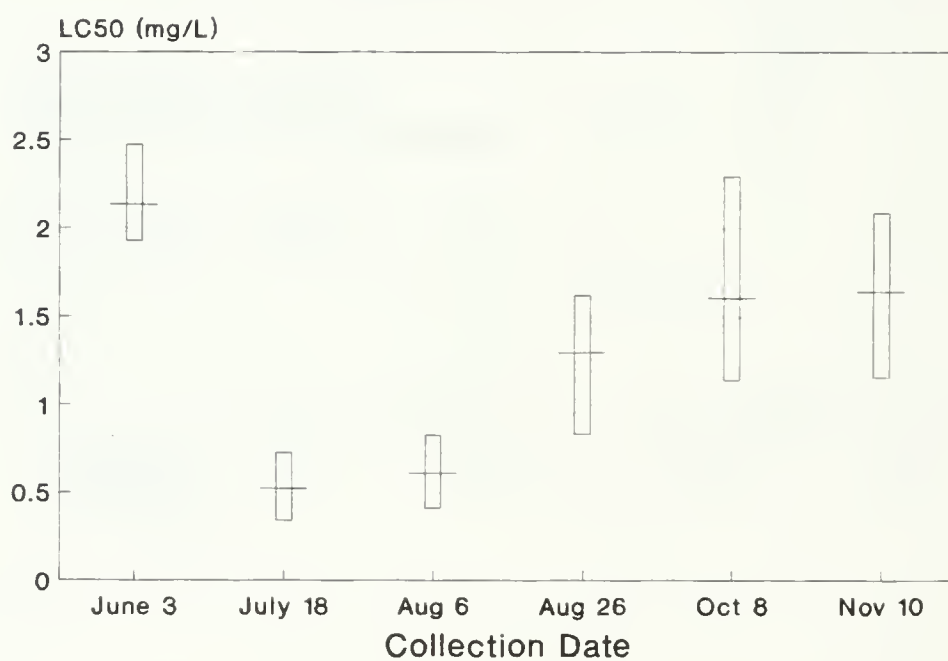


Figure 5. Seasonal variations in the sodium hypochlorite LC50 of zebra mussels from Lake St. Clair.



Figure 6. Seasonal variations in the bayluscide LC50 of zebra mussels from Lake St. Clair.

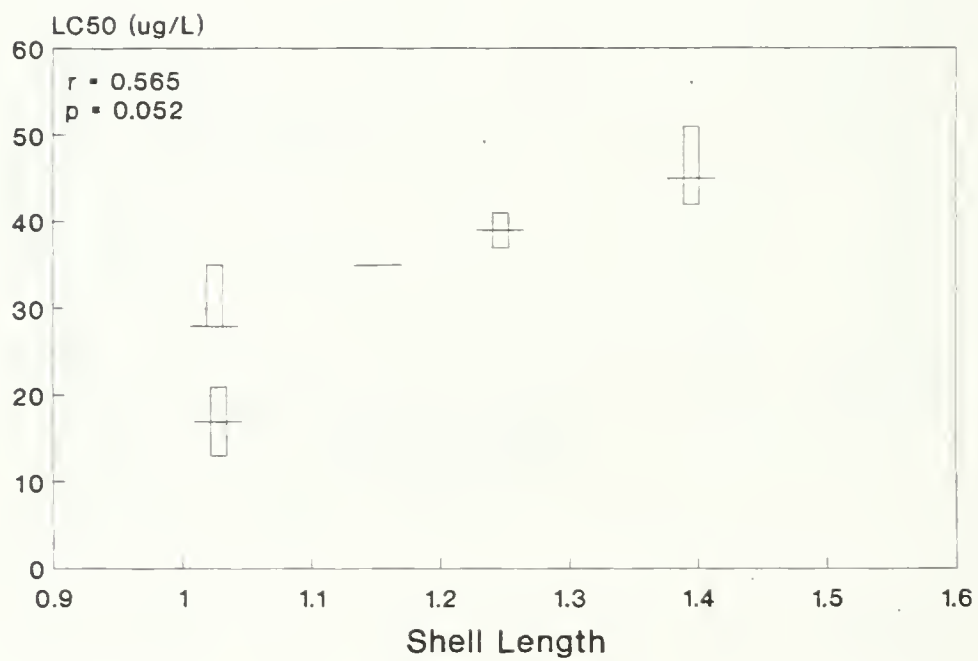


Figure 7. Relationship between the bayluscide LC50 and shell length of zebra mussels. Data are for mussels collected from Lake St. Clair at various times during the summer of 1992.

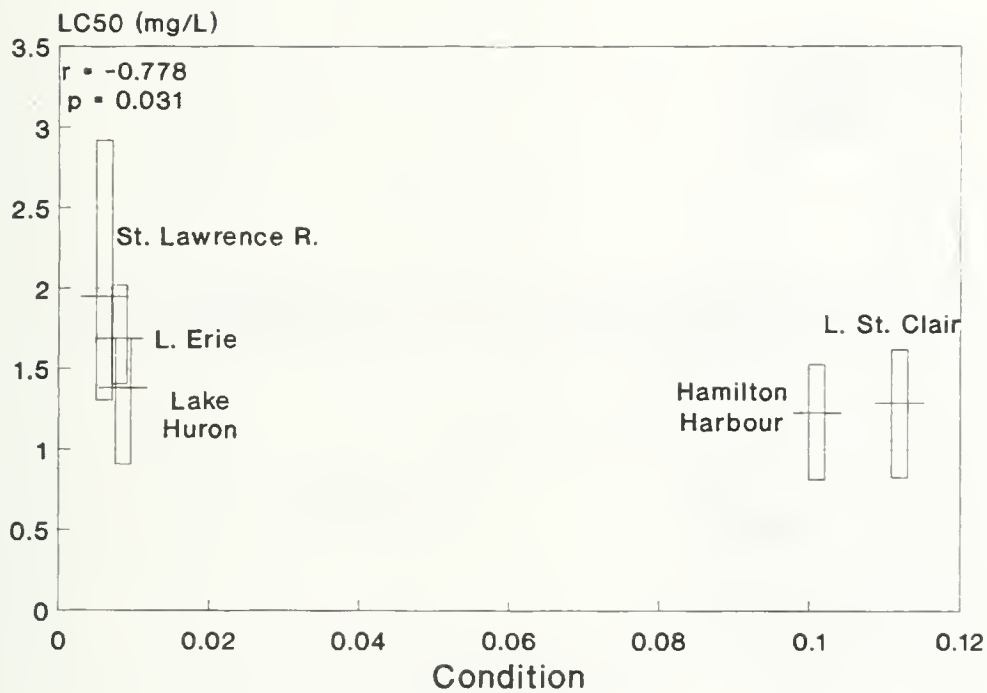


Figure 8. Relationship between the sodium hypochlorite LC50 and body condition of zebra mussels. Data are for mussels collected from different location during the week of August 24, 1992. Condition is expressed as Ash-Free-Dry-Weight for an average shell length.

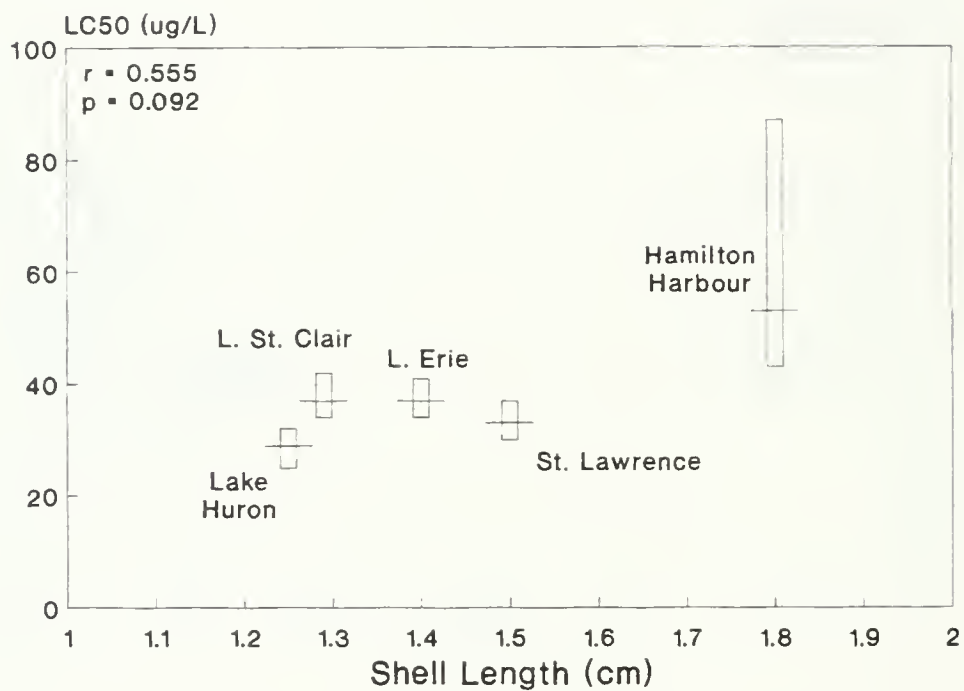


Figure 9. Relationship between the bayluscide LC50 and shell length of zebra. Data are for mussels collected from different locations during the week of August 24, 1992.

APPENDIX A

**List of the tests conducted and the sodium hypochlorite
and bayluscide LC50 values for adult zebra mussels.**

Table A.1. List of tests conducted and the sodium hypochlorite and bayluscide LC50 values for adult zebra mussels. AFDW refers to Ash-Free-Dry Weight (mg). AFDW values have been adjusted to a standard mussel 1.21 cm long (see Section 2.5).

Stock	Collection Date	Test Date	Effect Being Tested	AFDW	Shell Length (cm)	Hypochlorite LC50 (mg/L)	Bayluscide LC50 (μ g/L)
Puce	June 3/92	June 8/92	fed/starved 5 days	.0118	1.025	2.13 (1.93, 2.48)	
			fed/starved 5 days	.0125	1.00		28.0 (28.0, 35.0)
	June 9/92	June 9/92	Are new mussels different than mussels kept one week.		1.028		17.3 (14.1, 21.4)
	June 3/92	June 9/92	Are mussels maintained in the laboratory for one week different than fresh mussels.		0.971		6.4 (4.2, 8.9)
	June 3/92	June 15/92	fed 12 days		0.996		21.0 (21.0, 28.0)
			starved 12 days		1.10		21.0 (21.0, 28.0)
		June 29/92	fed 26 days	.0070	0.866		42.0 (28.0, 56.0)
			starved 26 days	.0108	0.963		42.0 (28.0, 56.0)
			fed 26 days	.0090	0.989	1.98 (1.29, 4.20)	
			starved 26 days	.0108	1.012	1.23 (0.92, 1.73)	
		July 6/92	starved 33 days	.0080	0.988		39.5 (33.3, 43.4)
			starved 33 days	.0094	1.06		47.1 (43.2, 52.1)
	July 18/92	July 22/92	seasonal effects	.0124	1.152		35.0 (35.0, 35.0)
			seasonal effects	.0124	1.152	0.53 (0.35, 0.73)	
	June 3/92	Aug 11/92	fed 59 days				47.5 (45.0, 50.3)
			starved 59 days				49.0 (42.0, 49.0)
			fed 59 days			1.34 (0.98, 1.80)	
			starved 59 days			2.41 (1.98, 1.80)	
	Aug 6/92	Aug 11/92	seasonal effects				44.0 (40.9, 48.2)

Stock	Collection Date	Test Date	Effect Being Tested	AFDW	Shell Length (cm)	Hypochlorite LC50 (mg/L)	Bayluscide LC50 (μ g/L)
seasonal effects						0.61 (0.41, 0.83)	
Hamilton	Aug 25/92	Aug 31/92	stock location	.0101	1.516		48.7 (45.3, 53.6)
			stock location	.0101	1.516	1.23 (0.82, 1.53)	
Puce	Aug 26		stock location	.0112	1.247		39.1 (37.3, 40.9)
			stock location	.0112	1.247	1.30 (0.83, 1.62)	
Port Dover	Aug 26		stock location	.0081	1.269		37.0 (33.9, 41.2)
			stock location	.0081	1.269	1.69 (1.41, 2.02)	
Cornwall	Aug 24		stock location	.0061	1.327		33.2 (30.2, 36.9)
			stock location	.0061	1.327	1.95 (1.31, 2.92)	
Goderich	Aug 27		stock location	.0086	1.228		27.8 (24.4, 31.0)
			stock location	.0086	1.228	1.38 (0.91, 1.69)	
Puce	Jun 3/92	Sep 8/92	starved 88 days	.0075	1.241		35.4 (33.5, 37.6)
			starved 88 days	.0067	1.152	1.62 (1.05, 2.80)	
			fed 88 days	.0075	1.22		29.4 (29.1, 31.0)
			fed 88 days	.0086	1.294	0.93 (0.63, 1.05)	
	Oct 8/92	Oct 13/92	large vs		1.508		<28.0 (all died) ¹
			small		0.475		<28.0 (all died)
			large vs		1.480	1.61 (1.14, 2.29)	
			small		0.429	1.13 (0.75, 1.60)	
	Nov 10/92	Nov 12/92	10°C/day baseline	.0122	1.383	1.34 (0.97, 1.86)	
			10°C/day baseline	.0122	1.383		41.0 (38.1, 44.5)
	Nov 10/92	Nov 17/92	acclimation rate 2°C/day		1.395	1.64 (1.16, 2.09)	

Stock	Collection Date	Test Date	Effect Being Tested	AFDW	Shell Length (cm)	Hypochlorite LC50 (mg/L)	Bayluscide LC50 (μ g/L)
			acclimation rate 10°C/day		1.631	2.11 (1.28, 2.25)	
			acclimation rate 2°C/day		1.395		45.2 (42.0, 50.5)
			acclimation rate 10°C/day		1.632		42.0 (42.0, 49.0)
		Dec 2/92	starved 180 d	.0078	1.044		23.2 (4.8, 27.0)
			starved 180 d	.0078	1.259	0.45 (0.23, 0.67)	
			fed 180 d	.0054	1.320	0.28 (0.22, 0.34)	
			fed 180 d	.0054	1.306		<40.0 no calculation ²
		Dec 5/92	starved 183 d	.0064	1.314		22.3 (18.9, 28.7)
			fed 183 d	.0224	1.572		25.9 (21.7, 37.8)

¹ In these experiments, all treated mussels died. The concentration given was the lowest concentration tested.

² In this experiment, there was 77% mortality in the lowest concentration tested (28 μ g/L) and 100% mortality in all other test concentrations. No calculation of the LC50 was possible with these data.

